ZooKeys 1180: 1–26 (2023)
DOI: 10.3897/zookeys.1180.108418

Research Article

Description of three new bat-associated species of hard ticks (Acari, Ixodidae) from Japan

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OPEN ACCESS

Academic editor: Vladimir Pesic
Received: 21 June 2023
Accepted: 12 August 2023
Published: 15 September 2023

ZooBank: https://zoobank.org/6112D084-4D9C-4A55-9AC2-C4F2B2245D49

Citation: Takano A, Yamauchi T, Takahashi M, Shimoda H, Gotoh Y, Mizuno J, Natsume M, Kontschán J, Kováts D, Tu VT, Hornok S (2023) Description of three new bat-associated species of hard ticks (Acari, Ixodidae) from Japan. ZooKeys 1180: 1–26. https://doi.org/10.3897/zookeys.1180.108418

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Abstract

In Eurasia, the geographically most widespread ixodid tick species of the bat families Rhinolophidae Gray, Vespertilionidae Gray, and Miniopteridae Dobson were considered to belong to four species, *Ixodes vespertilionis* Koch, *I. collaris* Hornok, *I. ariadnae* Hornok, and *I. simplex* Neumann. Previous data attest that bat-associated tick species from Eastern Asia show remarkable genetic difference from the above four tick species, but in the absence of detailed morphological comparison these were regarded as conspecific. In this study we compensate for this lack of data on three bat-associated tick species, reporting their morphological comparison, as well as molecular and phylogenetic relationships. According to the results we describe the females of three tick species new to science, i.e., *I. nipponrhinolophi* Hornok & Takano, **sp. nov.**, *I. fuliginosus* Hornok & Takano, **sp. nov.**, and *I. fujitai* Hornok & Takano, **sp. nov.** In case of all three new tick species the cytochrome *c* oxidase subunit (coxI) gene showed remarkably high sequence differences from the species that they previously were thought to belong to, well exceeding the average limit delineating ixodid tick species. This, as well as observed morphological differences fully justify their taxonomical status as new species.

Key words: Chiroptera, *Eschatocephalus*, Ixodida, long-legged bat tick, mitochondrial, 16S rRNA gene

Introduction

Bats (order Chiroptera) form the second largest order of mammals, with more than 1400 described species (Simmons and Cirranello 2023). Among mammals only bats are able to fly actively, which is an important factor behind their wide geographical distribution and seasonal migration (Hutterer et al. 2005). In part owing to their flight and their aggregated occurrence in colonies, bats frequently reach high population densities in or near urban habitats (Beltz 2018). During the past decades increasing chances of contact between bats and humans has been observed, due to increasing human introgression into natural bat habitats, as well as due to enhanced attraction of bats to agricultural facilities and human settlements where their food tends to abound (Han et al. 2015). Recent changes in bat behaviour also increase the risk of zoonotic transmission of pathogens by urbanisation as bats roost in artificial structures such as bridges and old mines, as well as homes, churches, schools, and barns (Beltz 2018). In addition, the total number of infectious bats may be higher in the urban, as opposed to rural, clusters (Plowright et al. 2011; Beltz 2018).

Bats play an important role in the epidemiology of vector-borne diseases. First and most importantly, the majority (approximately 70%) of all bat species are insectivorous (Simmons 2005) and due to this habit they have constant access to blood-sucking arthropod vectors as food items, possibly allowing mechanical transmission of relevant pathogens (Hornok et al. 2015a). Second, bats can also act as hosts of a broad range of haematophagous ectoparasites, among them biological vectors of pathogens (Klimpel and Mehlhorn 2014). Once infected with vector-borne pathogens, immunological adaptions of bats make them especially suitable reservoirs (Brook and Dobson 2015).

Among blood-sucking arthropod vectors, ticks (Acari: Ixodidae, Argasidae) are considered as the epidemiologically and ecologically most important in the temperate zone (Jongejan and Uilenberg 2004). Tick-infestation of bats may account for their infection with tick-borne pathogens, some of them zoonotic, as exemplified by borreliae, rickettsiae, and viruses (Oba et al. 2016; Beltz 2018). Bat ticks were also reported to carry DNA of pathogens (e.g., piroplasms) with high veterinary-medical importance (Hornok et al. 2016). In addition, the presence of ticks may affect the immunity to co-infection of bats with other pathogens (Beltz 2018). Last but not least, among bat-specialist tick species, the soft tick *Carios vespertilionis* Latreille (Argasidae), as well as two hard tick (Ixodidae) species, i.e., *Ixodes vespertilionis* Koch and *I. simplex* Neumann were also reported to opportunistically feed on humans (Jaenson et al. 1994; Piksa et al. 2013; Péter et al. 2021).

The taxonomy of bat-associated tick species has been subject to considerable revision during the past decade. First, a new ixodid bat tick species, *Ixodes ariadnae* Hornok was discovered in Europe (Hornok et al. 2014), followed by the description of another new bat-specialist tick species, *Ixodes collaris* Hornok, 2016 from Asia. Although it was demonstrated that further "short and long-legged" ixodid bat tick species differ genetically enough to justify their taxonomic status as separate species (Hornok et al. 2015b), until now these were not described due to lack of sufficient candidates as type specimens, especially adults. Therefore, the most important driver of the present study was to examine females of bat-associated tick collected in Japan, in order to evaluate their morphology and (if substantiated) to describe them as new species.

Materials and methods

Sample collection and ethical permission

Ticks were removed from bats or cave walls at various locations in Japan (Fig. 1; type material is described below). Ethical permission for bat capture was provided in Yamaguchi Prefecture (Prefectural Government Approval No. R3-429-1 and R4-165-1). After collection, the ticks were stored individually in vials containing 70% or 96% ethanol.

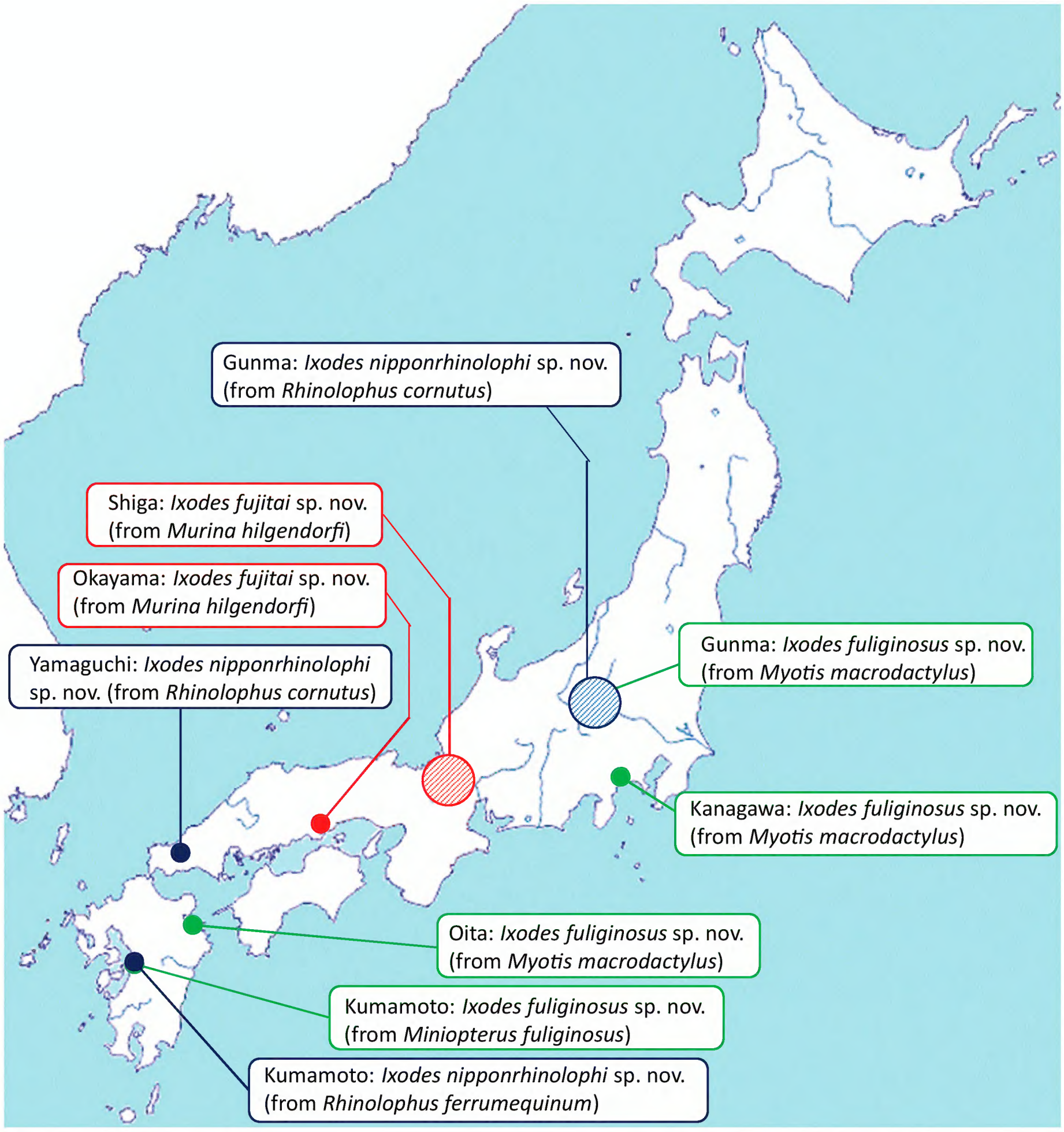
Gunma: *Ixodes nipponrhinolophi* sp. nov. (from *Rhinolophus cornutus*)
Shiga: *Ixodes fujitai* sp. nov. (from *Murina hilgendorfi*)
Okayama: *Ixodes fujitai* sp. nov. (from *Murina hilgendorfi*)
Yamaguchi: *Ixodes nipponrhinolophi* sp. nov. (from *Rhinolophus cornutus*)
Gunma: *Ixodes fuliginosus* sp. nov. (from *Myotis macrodactylus*)
Kanagawa: *Ixodes fuliginosus* sp. nov. (from *Myotis macrodactylus*)
Oita: *Ixodes fuliginosus* sp. nov. (from *Myotis macrodactylus*)
Kumamoto: *Ixodes fuliginosus* sp. nov. (from *Miniopterus fuliginosus*)
Kumamoto: *Ixodes nipponrhinolophi* sp. nov. (from *Rhinolophus ferrumequinum*)

Figure 1. Map of Japan showing the origin of type specimens examined in this study. The blue spots indicate *Ixodes nipponrhinolophi* Hornok & Takano, sp. nov., the green-coloured spots mark locations of *Ixodes fuliginosus* Hornok & Takano, sp. nov., and red shows *Ixodes fujitai* Hornok & Takano, sp. nov. Larger shaded spots indicate the region of origin if the precise location was not known.

Morphological analyses

Previously or recently collected female ticks (Suppl. material 1), which were morphologically intact, were selected for description of new species. Measurements were performed and pictures were taken with a VHX-5000 digital microscope including the software VHX-H4M 3D (Keyence Co., Osaka, Japan). The sizes in the descriptions below are provided in millimetres.

In addition, for each new species, at least one recently collected adult tick was also used for DNA extraction from one leg or cutting the idiosoma ventrally (i.e., to preserve them as voucher specimens). By contrast, males, nymphs, and larvae available during the study period were not suitable for morphological description (i.e., their DNA was already extracted or were severely damaged). Therefore, these were only used for DNA extraction (from their whole body) and molecular phylogenetic analyses. Conspecificity of males, nymphs and larvae with morphologically analysed female(s) was confirmed by at least one mitochondrial marker. Type specimens (female ticks) are summarised in Table 1, whereas all ticks used for either morphological and/or molecular analyses are shown in Suppl. material 1.

Table 1. Female tick specimens used for descriptions and comparisons. For new species, the holotypes are in cells with light purple background; paratypes are in cells with light grey background.

Complex	Tick species	Source of origin	Sample code	Collected by	Country (region or location) of collection	Date collected: year-month-day
I. vespertilionis	*Ixodes nipponrhinolophi*	*Rhinolophus cornutus*[4]	YB47	A.Takano	Japan (Mine city, Yamaguchi)	2021.02.25.
	Ixodes nipponrhinolophi[1]	*Rhinolophus ferrumequinum*	–	K. Funakoshi	Japan (Kuma-gun, Kumamoto)	1973.12.24.
	Ixodes nipponrhinolophi[1]	*Rhinolophus ferrumequinum*	–	K. Funakoshi	Japan (Kuma-gun, Kumamoto)	1979.04.12.
	Ixodes nipponrhinolophi[1]	*Rhinolophus ferrumequinum*	–	K. Funakoshi	Japan (Kuma-gun, Kumamoto)	1979.05.10.
	Ixodes nipponrhinolophi[1]	*Rhinolophus ferrumequinum*	–	K. Funakoshi	Japan (Kuma-gun, Kumamoto)	1979.05.25.
	Ixodes nipponrhinolophi	*Rhinolophus cornutus*	4673	M. Takahashi	Japan (Tano-gun, Gunma)	2005.04.23.
	Ixodes vespertilionis	Pálvölgyi Cave (wall)	KD36	D. Kováts	Hungary (Budapest)	2018.12.01.
	Ixodes vespertilionis	Leány Cave (wall)	CV1	D. Kováts	Hungary (Pilis Mountains)	2016.03.06.
	Ixodes vespertilionis	Leány Cave (wall)	CV142	D. Kováts	Hungary (Pilis Mountains)	2017.03.19.
	Ixodes collaris[2]	*Hipposideros pomona*	VN14-0011	V. Tan Tu	Vietnam (Kon Tum)	2014.09.22.
I. simplex	*Ixodes fuliginosus*	*Myotis macrodactylus*[5]	Kana2020	F. Sato	Japan (Sagamihara city, Kanagawa)	2022.09.30.
	Ixodes fuliginosus[1]	*Miniopterus fuliginosus*	–	K. Funakoshi	Japan (Kuma-gun, Kumamoto)	1979.04.12.
	Ixodes fuliginosus[1]	*Myotis macrodactylus*	–	K. Funakoshi	Japan (Hita-gun, Oita)	1997.07.17.
	Ixodes fuliginosus	*Myotis macrodactylus*	5997	M. Takahashi	Japan (Tone-gun, Gunma)	2014.06.17.
	Ixodes simplex	*Miniopterus schreibersii*	–	A. D. Sándor	Romania (Báziás)	2022.09.20-23.
I. ariadnae	*Ixodes fujitai*	unknown	–	H. Fujita	Japan (Inukami-gun, Shiga)	1990.12.09.
	Ixodes fujitai[3]	*Murina hilgendorfi*	Iv_0ka2013	M. Yamada	Japan (Eniwa city, Okayama)	2013.03.09.
	Ixodes fujitai	*Murina hilgendorfi*	–	K. Okumura	Japan (Inukami-gun, Shiga)	2016.04.22.
	Ixodes ariadnae	Legény Cave (wall)	CV86	D. Kováts	Hungary (Pilis Mountains)	2017.03.05.

[1] Published in Yamauchi and Funakoshi 2000; [2] published in Hornok et al. 2016; [3] published in Hornok et al. 2015; [4] molted from a nymph collected from this host; [5] originally collected from this host, preserved after egg-laying.

DNA extraction and coxI PCR analysis

DNA was extracted by DNeasy Blood and Tissue kit (QIAGEN, Germany). The partial fragments of cytochrome *c* oxidase subunit 1 gene (coxI), mitochondrial 16S rRNA gene (mt-rrs) and mitochondrial 12S rRNA gene (12S rDNA) were amplified using primers described in literature (Folmer et al. 1994; Ushijima et al. 2003; Beati and Keirans 2001; Norris et al. 1999). PCR was performed using Tks Gflex DNA Polymerase (TaKaRa Bio Inc., Japan). PCR products were purified with Axygen AxyPrep MAG PCR Clean-Up kit (Corning, USA) and sequenced by Eurofins Genomics Inc. (Tokyo, Japan). Obtained sequences were compared with GenBank data by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov).

Mitochondrial genome sequencing

Mitochondrial genomes (mt-genome) were sequenced as previously described (Kelava et al. 2021). Briefly, long-range PCR was performed with mtG_K23 and mtG_K26 primer pair using PrimeSTAR GXL DNA Polymerase (TaKaRa Bio Inc.). Short PCRs for gap filling were performed using primer pair: mtG_GapF1 (5'-AGG AAG CTT AAA TTC CTC GCA T-3') and mtG_GapR1 (5'-TGC CAG CCG CCG CGG TTA TAC A-3'). All PCR products were purified with Axygen AxyPrep MAG PCR Clean-Up kit (Corning) and sequenced by Illumina Miseq (Illumina, USA) for long-range PCR, and Eurofins Genomics Inc. for short-PCR products. The Illumina sequence libraries were constructed by NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England Biolabs) and were sequenced with the Illumina MiSeq platform with the paired-end sequence reads (300 bp ×2). The assembly was done by Platanus B ver. 1.3.2 (Kajitani et al. 2020). Mt-genome was annotated using Geneious Prime (Biomatters, Ltd., New Zealand). New sequences (LC769933–LC769956) were submitted to GenBank (Suppl. material 1).

Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method, Kimura 2-parameter model with the MEGA version 7.0 software (Kumar et al. 2016). Sequence datasets were resampled 1,000 times to generate bootstrap values. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Branch lengths are measured in the number of substitutions per site. The analysis involved 40 nucleotide sequences, and there were a total of 578 positions in the final dataset.

Depositories

The holotypes were used for measurements and (together with paratypes) for illustrations. The holotypes will be deposited in the Center for Collections, National Museum of Nature and Science, Tokyo, Japan. The paratypes will remain in the collections of Takeo Yamauchi and Mamoru Takahashi (Table 1).

Taxonomic account

Family Ixodidae Koch
Genus *Ixodes* Latreille
Subgenus *Eschatocephalus* von Frauenfeld

***Ixodes nipponrhinolophi* Hornok & Takano, sp. nov.**
https://zoobank.org/08621B6E-5F55-44DB-8DBE-C3B9C5363558
Figs 2, 3

Diagnosis. Medium size (female 4 mm long) brown tick. Legs and palps long. Scutum posteriorly broad, rounded, with moderately deep cervical grooves and pits. Hair covering dense both dorsally and ventrally. Coxae without spurs, coxae I and II with straight and III and IV with semicircular medial edges. Spiracular plates subcircular.

Material examined. *Holotype*: female, from *Rhinolophus cornutus* Temminck, Japan, Yamaguchi, Mine city, 34.251084°N, 131.243056°E (DD), 25 February 2021, A. Takano coll. ***Paratypes***: five females. (1): from *Rhinolophus ferrumequinum* (Schreber), Japan, Kumamoto, Kuma-gun, 32.252183°N, 130.651239°E (DD), 24 December 1973, K. Funakoshi coll. (2): female, from *Rhinolophus ferrumequinum*, Japan, Kumamoto, Kuma-gun, 32.252183°N, 130.651239°E (DD), 12 April 1979, K. Funakoshi coll. (3): female, from *Rhinolophus ferrumequinum*, Japan, Kumamoto, Kuma-gun, 32.252183°N, 130.651239°E (DD), 10 May 1979, K. Funakoshi coll. (4): female, from *Rhinolophus ferrumequinum*, Japan, Kumamoto, Kuma-gun, 32.252183°N, 130.651239°E (DD), 25 May 1979, K. Funakoshi coll. (5): female, from *Rhinolophus cornutus*, Japan, Gunma, Tano-gun, 36.086915°N, 138.721945°E (DD), 23 April 2005, M. Takahashi coll.

Morphology of female (holotype, unengorged). Length of the idiosoma (from the half point between scapular apices to the middle of posterior margin) 3.18, width 1.74, ratio of idiosomal length/width 1.83 (Suppl. material 2: fig. 1A).

Scutum elongated, tie-shaped, broadest near posterior third. Deepest point of concavity at anterior third of length (Fig. 2C-1). Length of scutum 1.62, maximum width 0.99, ratio of length/width 1.64. Scutum with cervical grooves deep, narrow in second quarter of length (Fig. 2C-2); scattered, small punctuations; and two pits close to its maximum width (Fig. 2C-3). Posterolateral edge (after maximum width) convex. Scutal setae few, close to scapulae 0.03 long.

Alloscutum with dense hair covering dorsally. Length of centrodorsal setae 0.1, marginodorsal setae 0.16. Idiosoma has dense hair covering ventrally. Genital aperture broad U-shaped, with nearly parallel end (Fig. 2D-1), slightly posterior to 2nd-to-3rd intercoxal space. Genital groove posteriorly converging, then diverging (Fig. 2D-2), bowling pin-shaped (Suppl. material 2: fig. 1B). Spiracular plates subcircular, diameter 0.44, aeropyles randomly distributed, width of aeropyle rows (minimum to maximum) 2:7; position of spiracle opening submarginal, rounded, diameter 0.06 (Suppl. material 2: fig. 1C). Anal valves with 4-4 setae, arranged in C-shaped curve (Suppl. material 2: fig. 1D). Anal groove slightly converging (Suppl. material 2: fig. 1B).

Length of gnathosoma (from palpal apices to posterior margin of basis capituli) 0.84, width of basis capituli dorsally 0.63. Ratio of gnathosomal length/basis capituli width 1.33. Basis capituli triangular, its sides anteriorly converging,

broadest at lateral ridge continuing ventrally (Fig. 2A-1), posteriorly as broad as at maximum width of palpal base, without cornuae; posterior margin dark, sclerotised, nearly linear. Shape of areae porosae triangular anteriorly, broad posteriorly (Fig. 2A-2), with short, low lateral ridge (Fig. 2A-3). Ventrally the basis capituli triangular anterior to maximum width, reverse trapezoidal posteriorly, with squared concavity in between. Auriculae absent (Fig. 2B-1).

Palps (dorsal view) long, club-shaped, length 0.7, broadest anterior to junction of segments II and III, maximum width 0.17, ratio of length/width 4.1. Palpal hairs 0.03–0.08, longest medially on palpal segment II. Palpal segment I. 0.08, palpal segment II. 0.4, palpal segment III. 0.23 long. Ratio of palpal segments II/III 1.8, segment II 2.8× longer than broad when viewed vertically; its "stalk" with surface in level with that of broad part (Fig. 2A-4).

Hypostome lanceolate, anterior tapering straight, apex pointed. Length 0.4, width 0.18, ratio of length/width 2.2. Dental formula 3/3 (mid-length), apically 4/4 or more. Teeth posteriorly long (0.06), slender, elevated (Fig. 2B-2).

Legs long and slender. All coxae marginally dark (sclerotised), without spines or spurs. Coxa I trapezoid, with minute hairs (posteriorly 0.05). Coxa II rectangular, medial edge straight (Fig. 2D-3), posteriorly wrinkled, posterolateral hairs short (0.1). Coxa III semicircular (Fig. 2D-4), hairs 0.03–0.07. Coxa IV semicircular, hairs 0.05. Tarsus I with long setae (< 0.25) dorsally, length 1.3, maximum diameter 0.15, ratio of length/diameter 8.7. Haller's organ elongated, with grouped (non-linear) anterior pit sensillae, one of them longer (0.04).

Differential diagnosis. *Ixodes nipponrhinolophi* Hornok & Takano, sp. nov. is easily distinguished from members of the *Ixodes simplex* complex based on the long legs, and from members of the *Ixodes ariadnae* complex according to its long palps. Within the *Ixodes vespertilionis* complex, differences in comparison with females of the most similar species, *Ixodes collaris* include the following characters of the latter. Anteriolaterally on the scutum, the deepest point of concavity is at the anterior 1/8 of its length (Fig. 3C-1); cervical grooves broad (Fig. 3C-2), there are no pits; punctuations dense, especially anteriolaterally, (Fig. 3C-3). Genital aperture horizontally C-shaped, with diverging end (Fig. 3D-1), between third coxae, genital groove posteriorly diverging (Fig. 2D-2; Suppl. material 3: fig. 2B). Spiracle opening elongated, diameter 0.1 (Suppl. material 4: fig. 3C). Anal groove parallel behind the anus (Suppl. material 3: fig. 2D). Basis capituli posteriorly with transverse ridge, continuing laterally then ventrally as broad "collar" (Fig. 3A-1); shape of areae porosae elongated, tapering both anteriorly and posteriorly (Fig. 3A-2), their border well-defined, elevated laterally as diverging, then converging high ridge (Fig. 3A-3). Ventrally collar broad, extending above first coxae parallel with their surface (Fig. 3B-1), unlike in *I. nipponrhinolophi* Hornok & Takano, sp. nov. Ratio of palpal segments II to III 1.6, segment II only 2.1-times longer than broad when viewed vertically; its broad part with surface elevated above that of "stalk" (Fig. 3A-4). Hypostome teeth posteriorly less elevated (Fig. 3B-2). Medial edge of coxa II slightly curved (Fig. 3D-3), of coxa III straight (Fig. 3D-4).

Gene sequences. The complete mitochondrial genome sequence was deposited in GenBank (LC769935). All accession numbers relevant to the new species are listed in Suppl. material 1.

Host records. Known host species: *Rhinolophus cornutus*, *Rhinolophus ferrumequinum*.

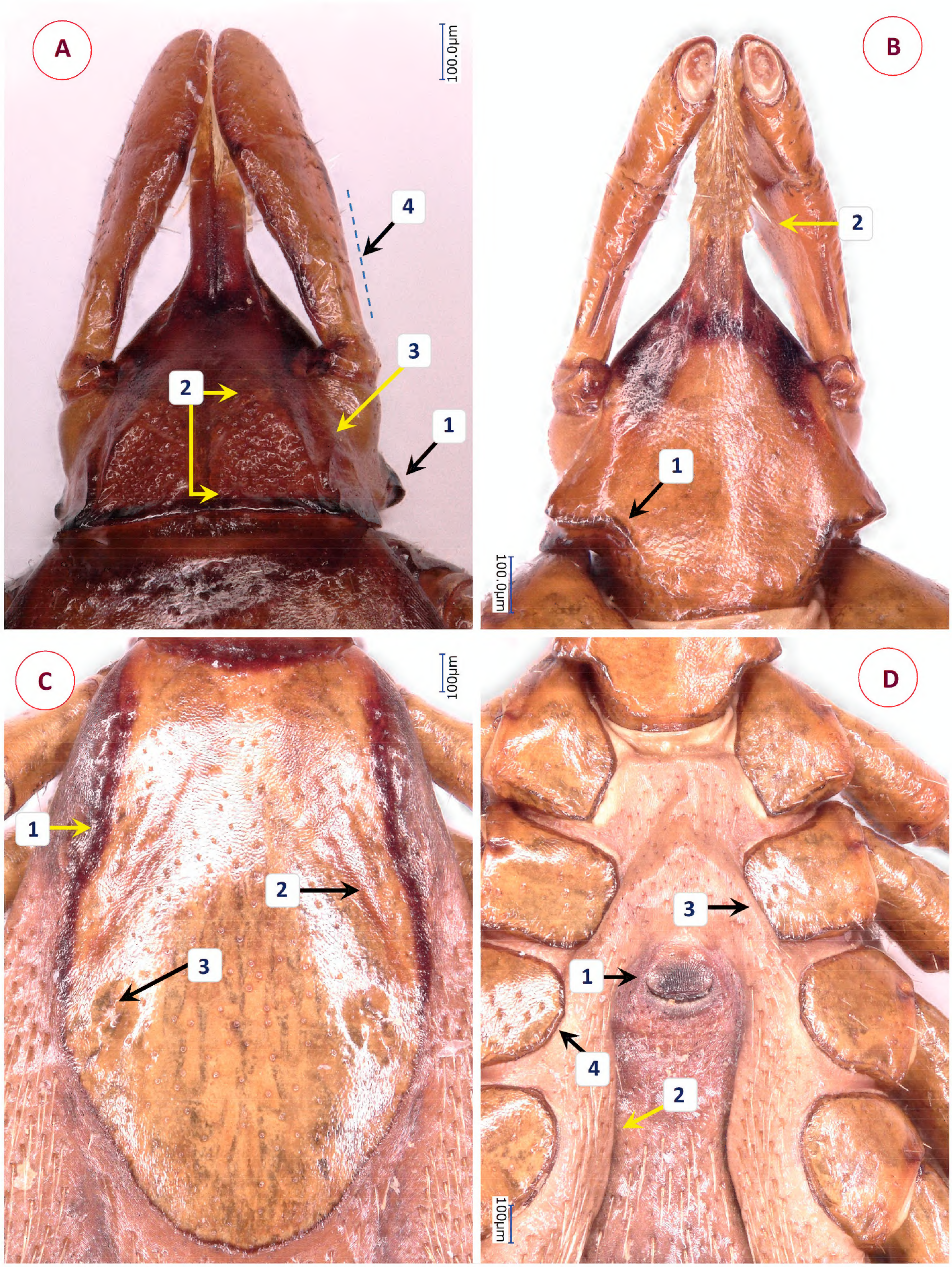

Figure 2. The morphology of *Ixodes nipponrhinolophi* Hornok & Takano, sp. nov.: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** idiosoma ventrally. Numbers are referred to in the text where relevant structures are described. Scale bars: 100 µm.

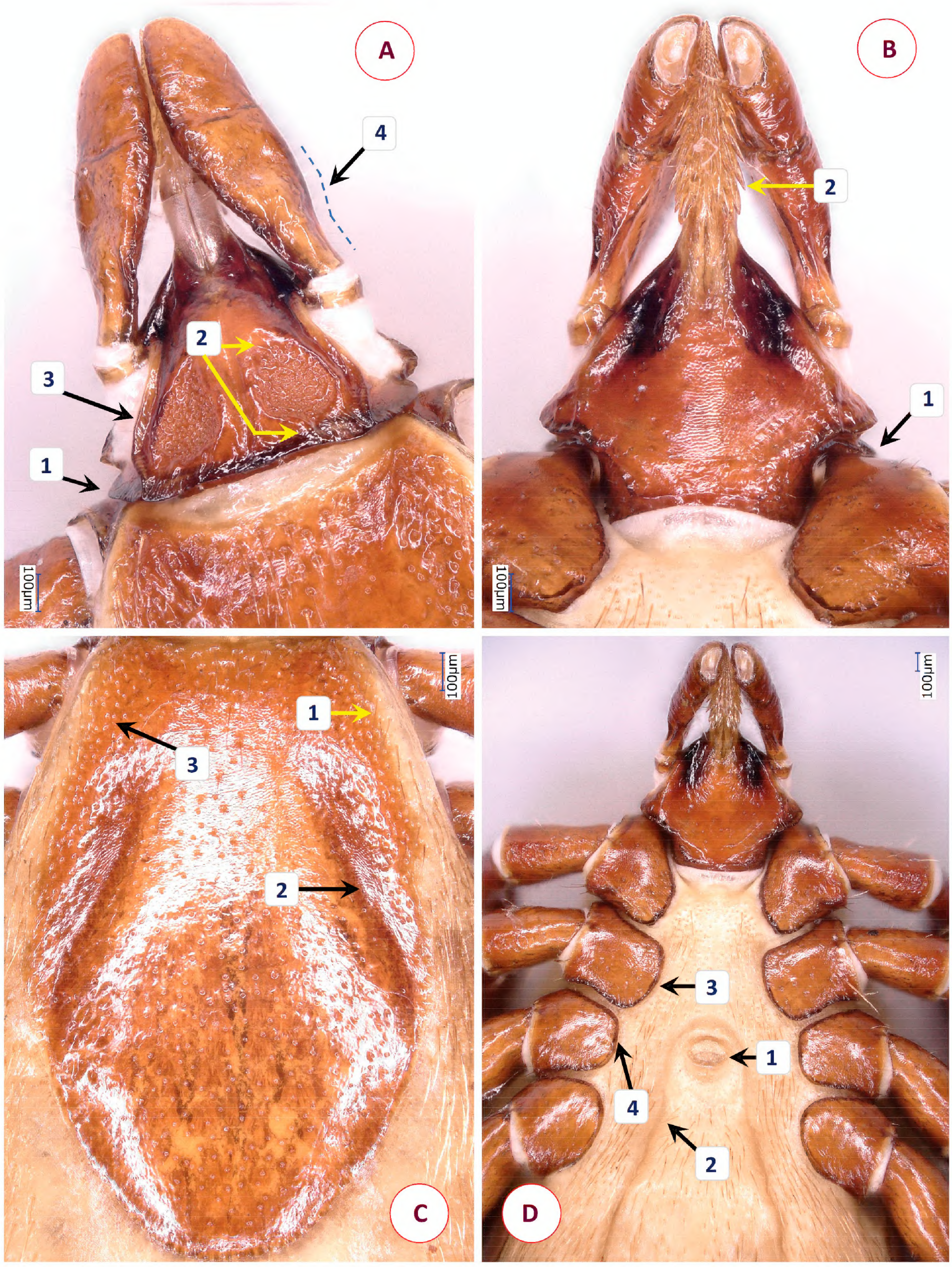

Figure 3. The morphology of *Ixodes collaris*: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** idiosoma ventrally. Numbers are referred to in the text where relevant structures are described. Scale bars: 100 µm.

Etymology. The name of the new species refers to Japan (in Japanese: Nippon), where all specimens have been collected, and to the type host family of horseshoe bats, Rhinolophidae.

***Ixodes fuliginosus* Hornok & Takano, sp. nov.**

https://zoobank.org/70478C08-894B-44EF-B307-40982FA22EFD

Figs 4, 5

Diagnosis. Medium size (female 3.3 mm long) brown tick. Legs and palps short. Scutum oval, shield-shaped. Hair covering dense dorsally but sparse ventrally. Coxae without spurs. Coxa IV with 16–18 hairs posteriorly. Spiracular plates ovoid, somewhat triangular.

Material examined. *Holotype*: female, from *Myotis macrodactylus* Temminck, Japan, Kanagawa, Sagamihara city, 35.623170°N, 139.165542°E (DD), 30 September 2022, F. Sato coll. ***Paratypes*:** three females. (1): from *Miniopterus fuliginosus* Hodgson, Japan, Kumamoto, Kuma-gun, 32.252183°N, 130.651239°E (DD), 12 April 1979, K. Funakoshi coll. (2): female, from *Myotis macrodactylus*, Japan, Oita, Hita-gun, 33.228090°N, 130.981712°E (DD), 17 July 1997, K. Funakoshi coll. (3): female, from *Myotis macrodactylus*, Japan, Gunma, Tone-gun, 36.685602 °N, 138.925637°E (DD), 17 June 2014, M. Takahashi coll.

Morphology of female (holotype, engorged). Length of the idiosoma (from the half point between scapular apices to the middle of posterior margin) 3.2, width 2.1, ratio of idiosomal length/width 1.5.

Scutum oval, shield-shaped, anteriolaterally concave (Fig. 4C-1; Suppl. material 4: fig. 3A), broadest slightly anteriorly to its mid-length. Mid-length of scutum 0.95, maximum width 0.79, ratio of length/width 1.2. Maximum width of scutum to interscapular distance ratio 1.86. Cervical grooves straight, broad, directed to mid-posterolateral edge (Fig. 4C-2). On the scutum scarce punctuations, except in cervical fields. Posterior edge rounded. Scutal setae medially, anteriolaterally 0.04–0.05.

Alloscutum has dense hair covering dorsally. Length of centrodorsal setae 0.13, marginodorsal setae 0.1 (near peritreme). Idiosoma has sparse hair covering ventrally. Genital aperture straight, between 2nd coxae (Suppl. material 4: fig. 3B-1). Genital grooves narrowest (as a "waist") between 3rd and 4th intercoxal spaces (Suppl. material 4: fig. 3B-2), slightly diverging (almost parallel) to mid-length between genital opening and anus, then sharply diverging. Spiracular plates ovoid, diameter 0.24, aeropyles in rows (minimum to maximum) of 3–8; position of spiracle opening submarginal, rounded, diameter 0.03 (Suppl. material 4: fig. 3C). The shape of spiracular plates may have individual variation (Suppl. material 4: fig. 3D). Anal groove curved at front, slightly diverging behind the anus (Suppl. material 4: fig. 3B-3).

Length of gnathosoma (from palpal apices to posterior margin of basis capituli) 0.47, width of basis capituli dorsally 0.35. Ratio of gnathosomal length to basis capituli width 1.34. Basis capituli pentagonal, its sides parallel, posterior margin nearly straight (Fig. 4A-1), without cornuae and posteriolateral corner blunt. Basis length 0.16, width 0.37, length to width ratio 0.43. Front of basis nearly horizontal when joining the hypostome, enclosing an angle of ~ 120° (Fig. 4A-2). Shape of areae porosae triangular, their surface in level with

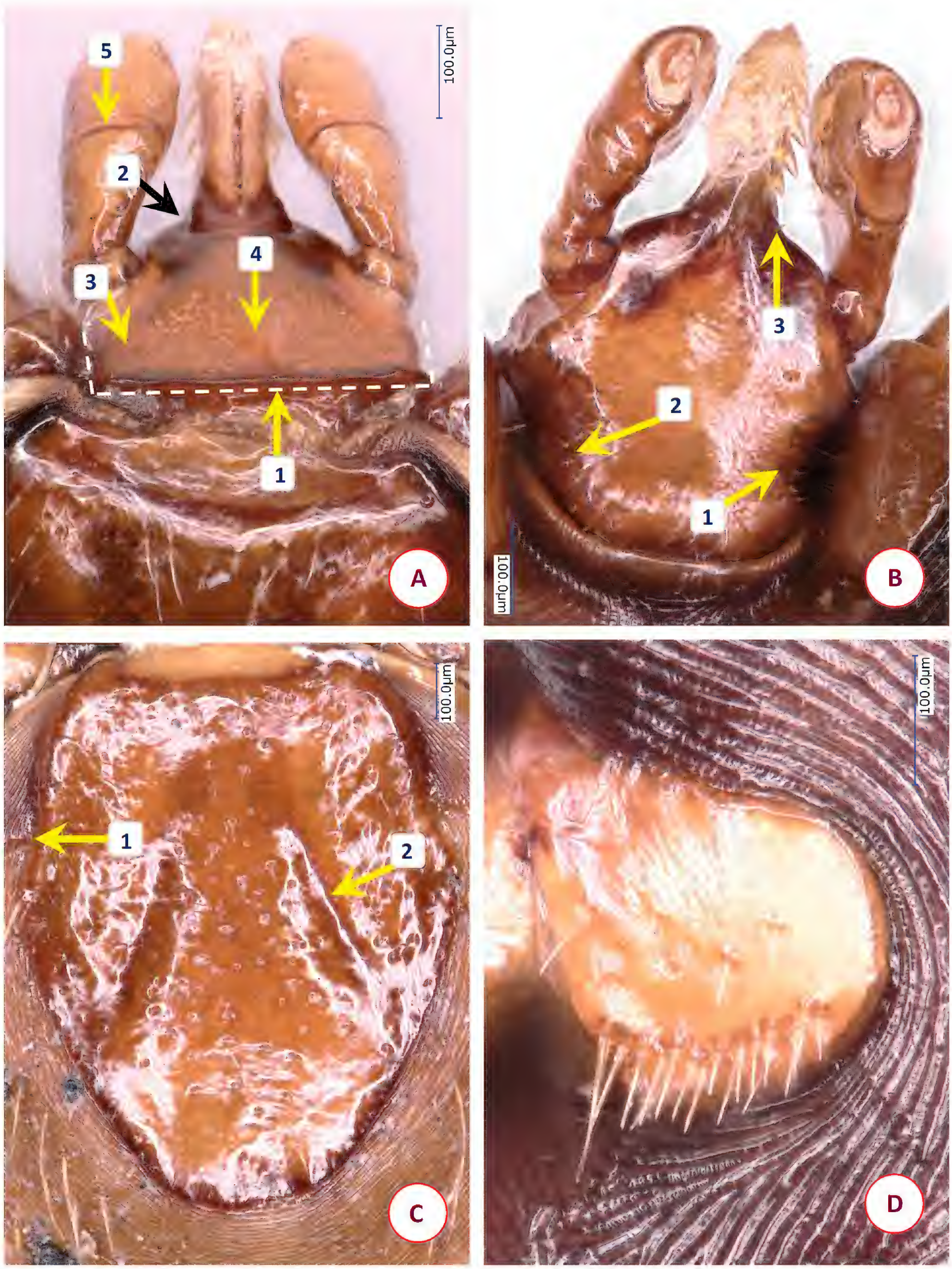

Figure 4. The morphology of *Ixodes fuliginosus* Hornok & Takano, sp. nov.: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** coxa IV. Numbers are referred to in the text where relevant structures are described. Dashed line indicates the edge of basis capituli. Scale bars: 100 µm.

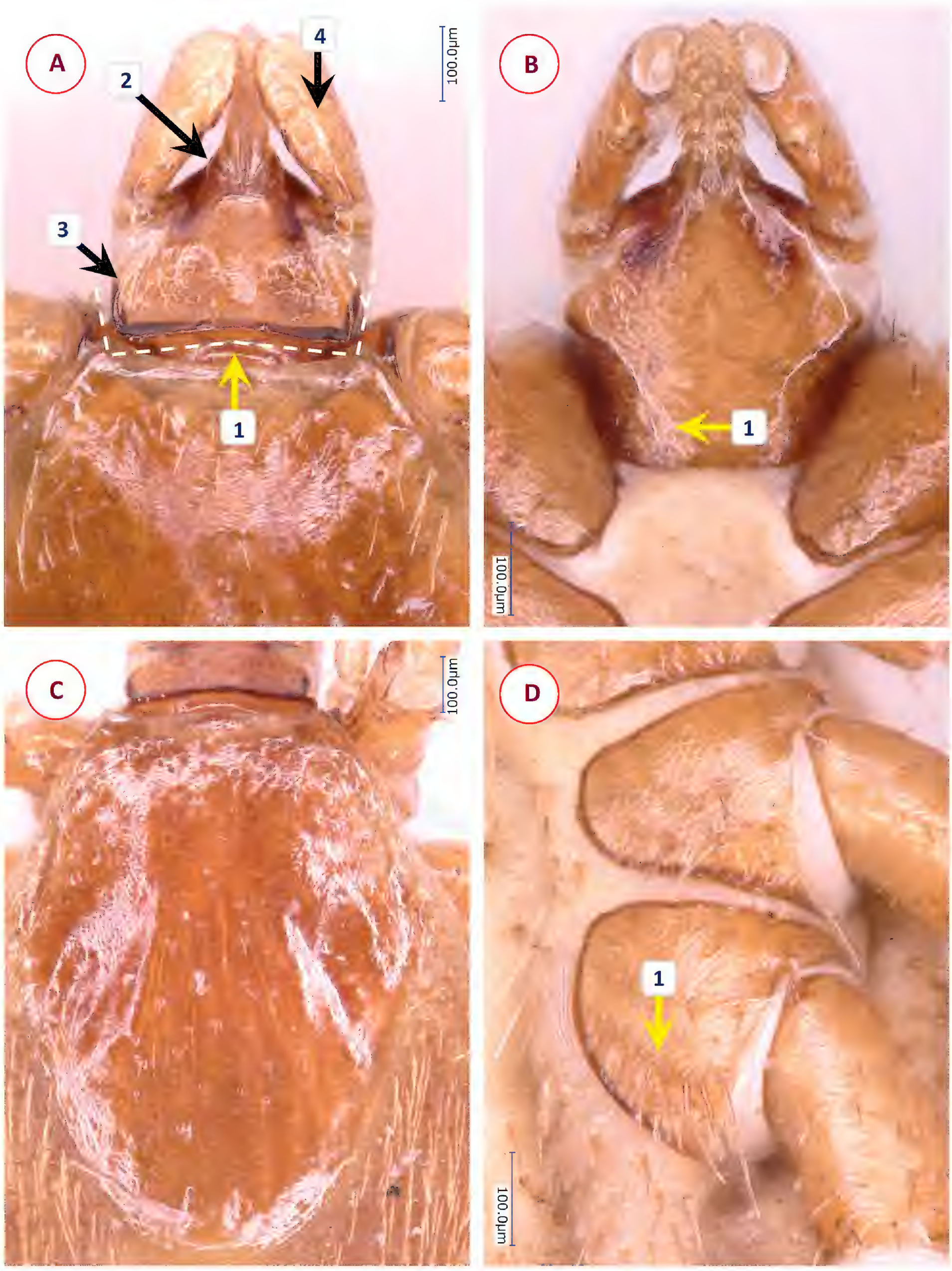

Figure 5. The morphology of *Ixodes simplex*: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** coxae III and IV. Numbers are referred to in the text where relevant structures are described. Dashed line indicates the edge of basis capituli. Scale bars: 100 µm.

surrounding smooth surface of basis (Fig. 4A-3). Laterally to areae porosae broad, smooth margin, similar in width (0.05) to interval between them (Fig. 4A-4). Ventrally basis capituli broad both anteriorly and posteriorly, with a narrowing ("waist") between (Fig. 4B-1), thus forming a figure-of-eight shape, posteriolaterally with hair covering (length 0.01) (Fig. 4B-2). Auriculae absent, auricular ridges short and inapparent. Posthypostomal setae medially, laterally 1-1 pair (length 0.02) (Fig. 4B-3).

Palps (dorsal view) short, club-shaped, anteriorly rounded, edge curved medially, straight laterally, length 0.33, maximum width 0.13, ratio of length/width 2.5. Joining of palpal segments II and III clearly visible as dark line (Fig. 4A-5). Palpal hairs few, numbering five or six laterally, dorsally, and medially; anteriorly short (0.015), longest medially on segment II (0.035). Palpal segment II triangular, 0.14 long. Palpal segment III same length (0.14).

Hypostome apex blunt, length 0.2, width 0.1, ratio of length/width 2. Dental formula 2/2 distally, 3/3 proximally (2-2 rows).

Legs short. All coxae medially rounded, without spines or spurs. Coxae I trapezoid, with three hairs (length 0.06–0.13). Coxa II with three hairs, coxa III with eight hairs, coxa IV with a tuft of 16–18 posterior hairs (0.035–0.125) (Fig. 4D). Tarsus I. length 0.73, maximum diameter 0.16, length to diameter ratio 4.5. Tarsal hairs short (0.03–0.05), except one long dorsal pair (0.17).

Differential diagnosis. *Ixodes fuliginosus* Hornok & Takano, sp. nov. can be easily distinguished from members of the *I. vespertilionis* and *I. ariadnae* complexes based on its short legs (length/maximum diameter ratio below 5, vs above 8 in long-legged bat ticks). Differences in comparison with females of the most similar species, *Ixodes simplex* include the following characters of the latter. More elongated, rhombus-shaped scutum (Suppl. material 5: fig. 4A), with length/width ratio 1.3 or above (vs ~ 1.2 in *I. fuliginosus* Hornok & Takano, sp. nov.) (Fig. 5C). Maximum width of scutum/interscapular distance ratio 2.16 (vs 1.86 in *I. fuliginosus* Hornok & Takano, sp. nov.). Position of genital aperture more posterior, between 3rd and 4th intercoxal spaces (Suppl. material 5: fig. 4B-1), and genital groove narrowest between 4th coxae (Suppl. material 5: fig. 4B-2). Long hair (0.1) anterior to and encircling the genital pore of *I. simplex* (Suppl. material 5: fig. 4B), absent on holotype and paratypes of *I. fuliginosus* Hornok & Takano, sp. nov. Basis capituli sides forwardly diverging then converging, posterior edge concave (Fig. 5A-1), without cornuae but posteriolateral corner sharp. Basis length 0.22, width 0.33, length to width ratio 0.67 (i.e., basis more elongated in *I. simplex* than in *I. fuliginosus* Hornok & Takano, sp. nov.). Front of basis steeply sloping when joining the hypostome, enclosing an angle larger (~ 135°) in *I. simplex* than in *I. fuliginosus* Hornok & Takano, sp. nov. (Fig. 5A-2). In *I. simplex* lateral margins of areae porosae elevated as ridge (Fig. 5A-3), much narrower than interval between areae, whereas in *I. fuliginosus* Hornok & Takano, sp. nov. lateral margins are smooth and broad. The separation of palpal segments II and III indistinct in *I. simplex* (Fig. 5A-4), unlike in *I. fuliginosus* Hornok & Takano, sp. nov. Ventrally the basis is rhombus-shaped, posteriorly converging in *I. simplex* (Fig. 5B-1) unlike in *I. fuliginosus* Hornok & Takano, sp. nov. The number of posterior setae (hairs) is fewer, 8–12 on coxae IV in *I. simplex* (Fig. 5D-1). The spiracular plates and the slightly diverging anal grooves are similar in these two species (Suppl. material 5: fig. 4C, D).

Gene sequences. Complete mitochondrial genome sequence was deposited in the GenBank (LC769933). All accession numbers relevant to the new species are listed in Suppl. material 1.

Host records. Known host species: *Miniopterus fuliginosus*, *Myotis macrodactylus*.

Etymology. The name of the new species refers to its bat host species of the genus *Miniopterus* which occurs in Japan (the type species of the *I. simplex* complex to which *I. fuliginosus* Hornok & Takano, sp. nov. belongs is a specific parasite of this genus of bats).

***Ixodes fujitai* Hornok & Takano, sp. nov.**

https://zoobank.org/AFE18817-243E-4793-B1E3-D49C02D7C7C8

Figs 6, 7

Diagnosis. Medium size (engorged female 6.8 mm long) yellowish tick. Legs long, palps short, areae porosae large. Scutum anteriorly trapezoid, posterolateral edge straight then rounded convex with wrinkled surface along its margin. Hair covering is sparse both dorsally and ventrally.

Material examined. *Holotype*: female, from unknown host species, Japan, Shiga, Inukami-gun, 35.222448°N, 136.291747°E (DD), 9 December 1990, H. Fujita coll. ***Paratypes*:** two females. (1): from *Murina hilgendorfi* Peters, Japan, Okayama, Eniwa city, 34.961817°N, 133.631483°E (DD), 3 September 2013, K. Funakoshi coll. (2): female, from *Murina hilgendorfi*, Japan, Shiga, Inukami-gun, 35.222448°N, 136.291747°E (DD), 22 April 2016, K. Okumura coll.

Morphology of female (holotype, engorged). Length of the idiosoma (from the half point between scapular apices to the middle of posterior margin) 6.8, width 4.8, ratio of idiosomal length/width 1.42.

Scutum broad pentagonal (broadest slightly anterior to mid-length), anterior part trapezoid, posterolateral edge straight then rounded convex with wrinkled surface along its margin (Fig. 6C). Length of scutum 1.2, maximum width 1.03, ratio of length/width 1.17. Posterolateral, converging margin of scutum straight in ~ one 1/4 of scutal length. On the scutum curved, deep cervical grooves reaching posterolateral margin in its anterior part, i.e., at the end of straight edge (behind maximum width), with slight concavity (Fig. 6C-1). Punctuations moderately sparse, large. Dorsal scutal setae few in middle (length 0.015).

Alloscutum with sparse hair covering dorsally. Length of centrodorsal setae 0.125, idiosoma has sparse hair covering ventrally. Genital aperture between 2nd and 3rd intercoxal spaces, as broad horizontal C-shape. Genital groove posteriorly diverging, with narrowing at level of 4th coxae. Spiracular plates subcircular, diameter 0.38, opening elongate (0.06), close to margin (Fig. 6D). Aeropyles in (2–3):(8–9) rows (minimum: maximum), densely packed, with margin broader than diameter of opening. Anal valves with two long (0.13) posterior setae. Anal groove slightly converging behind anus.

Length of gnathosoma (from palpal apices to posterior margin of basis capituli) 0.66, width of basis capituli dorsally 0.48. Ratio of gnathosomal length to basis capituli width 1.38. Basis capituli pentagonal (Fig. 6A), its sides anteriorly slightly then sharply converging. At the hypostomal basis dorsal flattening (plateau) (Fig. 6A-1), anteriorly gradually tapering to hypostomal base. Posterior

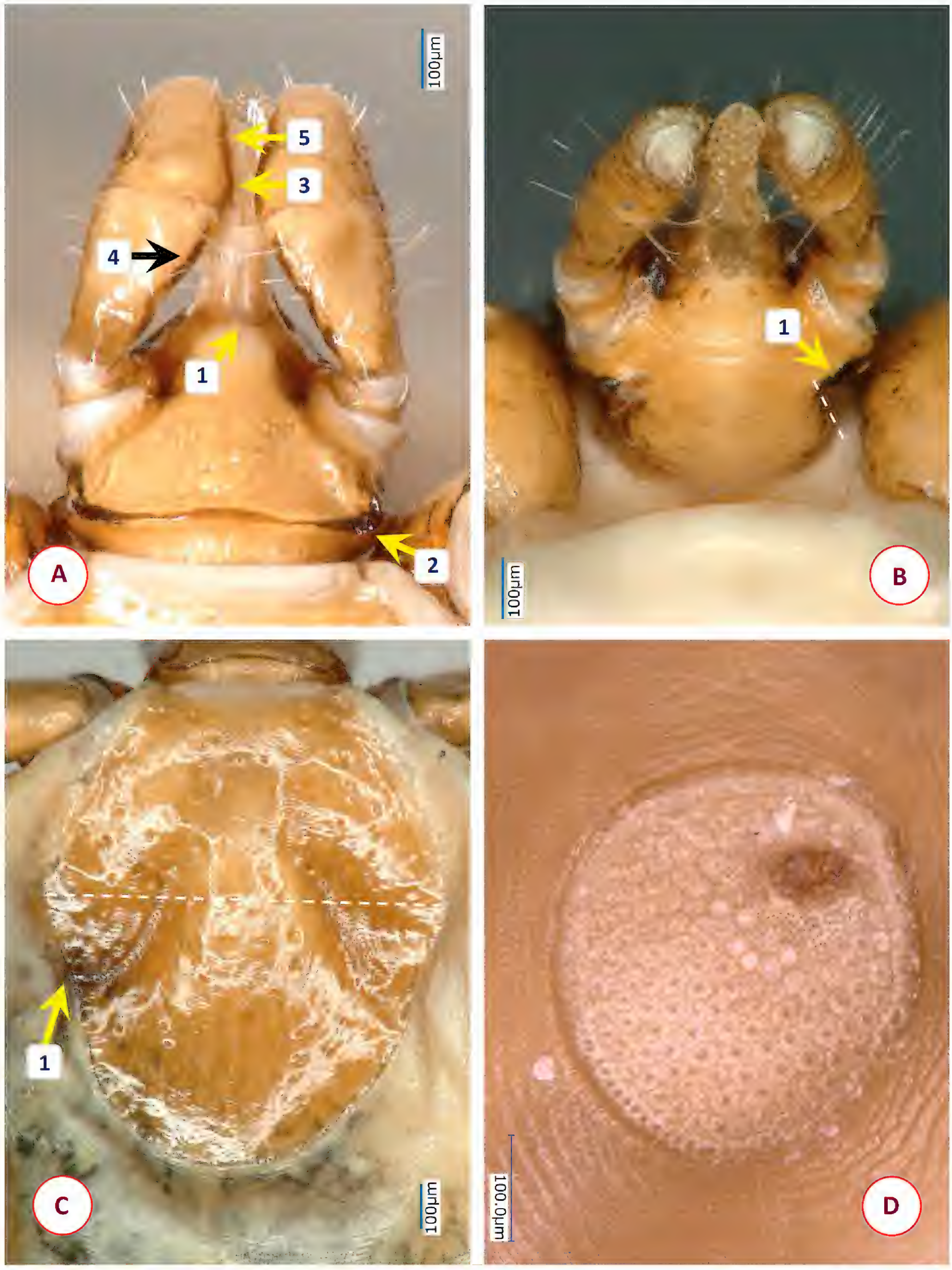

Figure 6. The morphology of *Ixodes fujitai* Hornok & Takano, sp. nov.: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** spiracular plate. Numbers are referred to in the text where relevant structures are described. Dashed lines indicate in **B** the posterolateral edge of ventral basis capituli and in **C** the maximum scutal width. Scale bars: 100 µm.

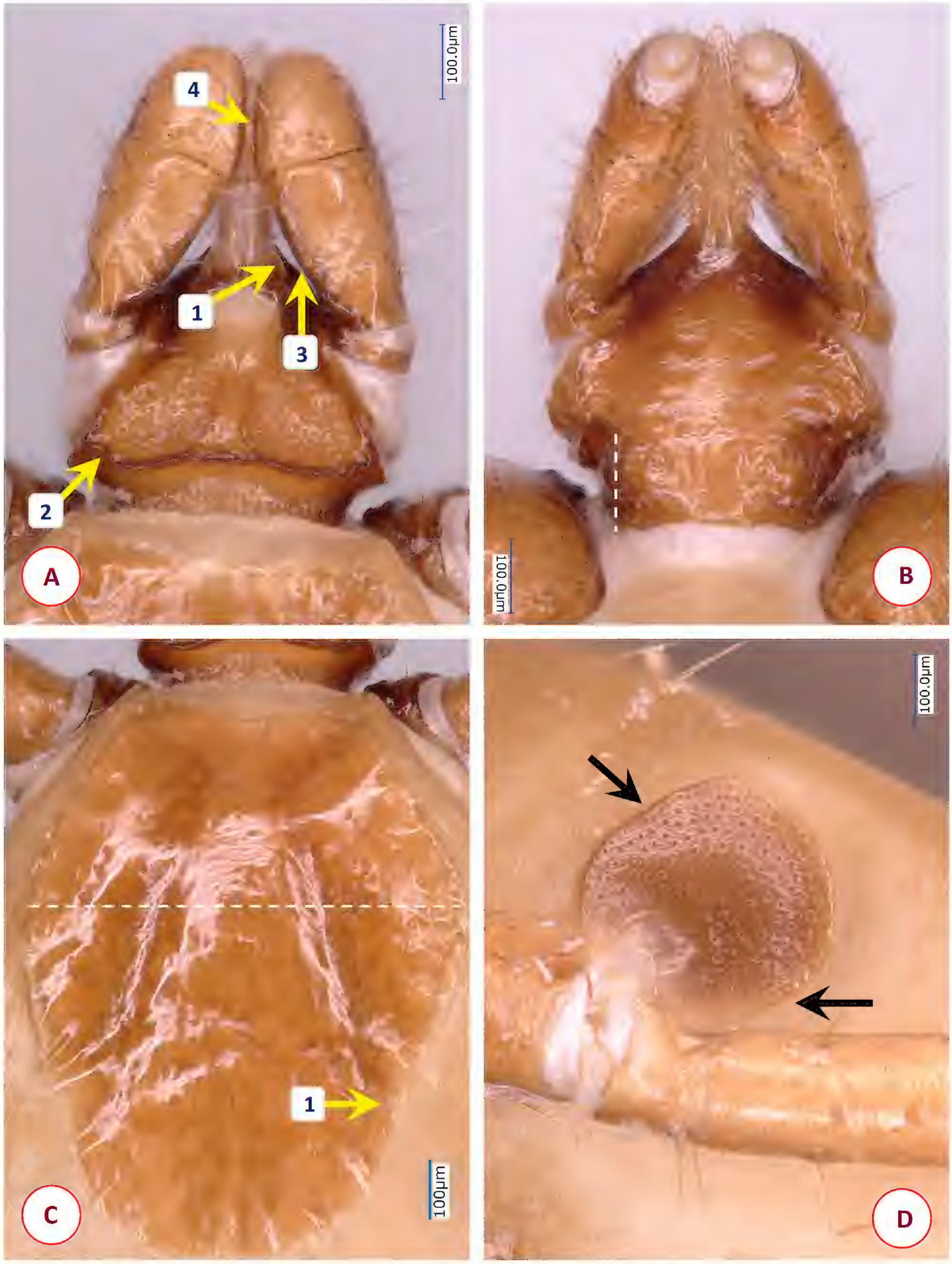

Figure 7. The morphology of *Ixodes ariadnae*: **A** dorsal aspect of basis capituli **B** ventral aspect of basis capituli **C** scutum **D** spiracular plate. Numbers are referred to in the text where relevant structures are described. Dashed lines indicate in **B** the posterolateral edge of ventral basis capituli and in **C** the maximum scutal width. Scale bars: 100 µm.

margin wavy, slightly concave in middle, without cornuae but with squared, thickened corners (Fig. 6A-2). Areae porosae large, posterolaterally tapering, their interval 0.08. Ventrally basis capituli converging along straight, dark auricular ridges (Fig. 6B-1) to narrowing ("waist"), posteriorly diverging (Fig. 6B, dashed line).

Palps (dorsal view) short, club-shaped, broadest anterior to junction of segments II and III as a protuberance medially (Fig. 6A-3), anteriorly rounded. Edge as a broken line medially (both palpal segments II and III with relatively straight medial edge) (Fig. 6A-4, -5), straight laterally. Length 0.52, maximum width 0.19, ratio of length/width 2.7. Palpal hairs number 16–18 laterally (length 0.05–0.1), anteriorly five or six short (up to 0.05), medially few (0.02–0.05). Palpal segment II 0.29 long, palpal segment III 0.19 long, their ratio 1.5.

Hypostome broken on holotype (absent on paratype). Dental formula 2/2 where visible.

Legs long and slender. Coxa I subtriangular, coxae II and III trapezoidal with rounded medial edge (coxa II straight in mid-length), coxa IV semicircular. On coxae III and IV caudolateral long hair measuring 0.20–0.22. Tarsus I length 1.5, maximum diameter 0.17, length/diameter ratio 8.8. Hairs on tarsus I dorsally, laterally, and ventrally 0.07–0.30. Haller's organ elongated, with grouped (non-linear) three anterior pit sensillae, one longer and stout (0.05).

Differential diagnosis. *Ixodes fujitai* Hornok & Takano, sp. nov. can be easily distinguished from *I. simplex* based on its long legs (length to maximum diameter ratio above 8), and from members of the *I. vespertilionis* complex based on its short palps. Differences in comparison with females of the most similar species, *Ixodes ariadnae* include the following characters of the latter. In *I. ariadnae* the scutum is slightly more elongated (ratio of length/width above 1.25 vs below 1.2 in *I. fujitai* Hornok & Takano, sp. nov.) (Fig. 7C). Posterolateral, converging margin of scutum in *I. ariadnae* straight in more than one third of scutal length (i.e., this part is longer than in *I. fujitai* Hornok & Takano, sp. nov.). On the scutum of *I. ariadnae* deep cervical grooves are straight in most of their length, reaching posterolateral margin in its middle, with more pronounced concavity (Fig. 7C-1). Punctuations on scutum very sparse. Spiracular plates of *I. ariadnae* subcircular but with straight portions of its edges (Fig. 7D), diameter smaller (0.33 vs 0.38 in *I. fujitai* Hornok & Takano, sp. nov.). Aeropyles loosely packed, with narrower margin than the diameter of their opening. Hypostome has laterally thickened basis (where sides are parallel with longitudinal axis) (Fig. 7A-1). Caudolateral corner of dorsal basis blunt, oblique (Fig. 7A-2) (vs squared, thickened angle in *I. fujitai* Hornok & Takano, sp. nov.). Posterior margin of basis wavy, strongly concave in middle (vs slightly in *I. fujitai* Hornok & Takano, sp. nov.). Ventrally basis capituli converging to narrow "waist" posterior to which it is nearly parallel (Fig. 7B dashed line); auricular ridge barely visible, scarcely sclerotised. In *I. ariadnae*, at the junction of palpal segments II and III no protuberance medially; their medial edge curved (Fig. 7A-3, -4) (vs straighter in *I. fujitai* Hornok & Takano, sp. nov.).

Gene sequences. Complete mitochondrial genome sequence was deposited in the GenBank (LC769934). All accession numbers relevant to the new species are listed in Suppl. material 1.

Host record. Known host species: *Murina hilgendorfi*.

Etymology. The name of the new species refers to the collector of holotype, the late Dr. Hiromi Fujita (Ohara General Hospital), who was a leading researcher and major author on the topic of ticks and tick-borne pathogens.

Discussion

Previously, when the molecular taxonomy of bat-specialist ticks was surveyed in Eurasia, the results indicated that several species new to science might exist among them in Asia (Hornok et al. 2015b). However, bat ticks are rare in Japan, in part because the percentage of parasitised bats is usually low (Yamauchi and Funakoshi 2000). Nevertheless, during the past years collections of bat ticks continued and in the present study females were examined both morphologically and molecularly, to describe three candidates as new species.

Considering molecular genetic comparison of ixodid ticks in general, the homologies of coxI sequences were reported to be above 93.9% within species, and below 94.4% between species (Lu et al. 2013). In line with this, the proposed average sequence divergence between closely related species (as a reference value delineating species) is 6.1% in the coxI, the so-called barcoding gene (Lv et al. 2014). This was considered when comparing the coxI sequences of each new species with its closest relative in a phylogenetic context (Fig. 8).

Ixodes nipponrhinolophi Hornok & Takano, sp. nov. was formerly considered *I. vespertilionis*. In the morphological illustration of the latter species collected in Japan (Yamaguti et al. 1971), certain morphologic characters were shown to be different from *I. vespertilionis* occurring in Europe (e.g., the arrangement of sensillae in Haller's organ) and other characters as different from *I. collaris* described from Vietnam (e.g., the shape of scutum and the basis capituli ventrally). Phylogenetically, the closest relative of *I. nipponrhinolophi* Hornok & Takano, sp. nov. is *I. collaris* (Fig. 8). Based on coxI sequences, the genetic divergence between these two species is in the range of 11.9–12.5% (74–78/624 bp) which exceeds (doubles) the average limit of interspecific boundary, 6.1% (Lv et al. 2014). This, as well as the morphological differences described above, fully justify the taxonomical status of *I. nipponrhinolophi* Hornok & Takano, sp. nov. as a new species.

Regarding bat species, one of the most important hosts of *I. nipponrhinolophi* Hornok & Takano, sp. nov. in Japan is the Greater Horseshoe bat, *Rhinolophus ferrumequinum* (Arthur 1956; Yamaguti et al. 1971). This species is widely distributed across Eurasia, from south-western Europe to Japan (Frutos et al. 2021). By contrast, the preferred host species of *I. collaris*, the Intermediate Horseshoe bat, *R. affinis* Horsfield, 1823 (Hornok et al. 2019) occurs only in Southeast Asia (Frutos et al. 2021).

Moreover, within *I. nipponrhinolophi* Hornok & Takano, sp. nov., the coxI sequence identity between specimens collected in southwestern Japan (Yamaguchi prefecture) and central Japan (Gunma prefecture) is very similar in magnitude (98%) to the difference reported between *I. vespertilionis* from Western Europe (France) and Central Europe (Hungary). In both cases there is a geographical barrier to bat migration and no direct contact between relevant bat populations, i.e., the Alps in Europe and the Alps in Japan, providing the most likely explanation for this genetic difference and reproductive isolation behind this phenomenon.

Long-known records and illustrations of *I. vespertilionis* or closely related species from various regions allow the conclusion on its occurrence in Asia. Thus, based on the morphology of the scutum and basis capituli, *I. vespertilionis* (and not *I. collaris* or *I. nipponrhinolophi* Hornok & Takano, sp. nov.) was reported from Russia (Pomerantzev 1950) and China (Kuofan and Zaijie 1991).

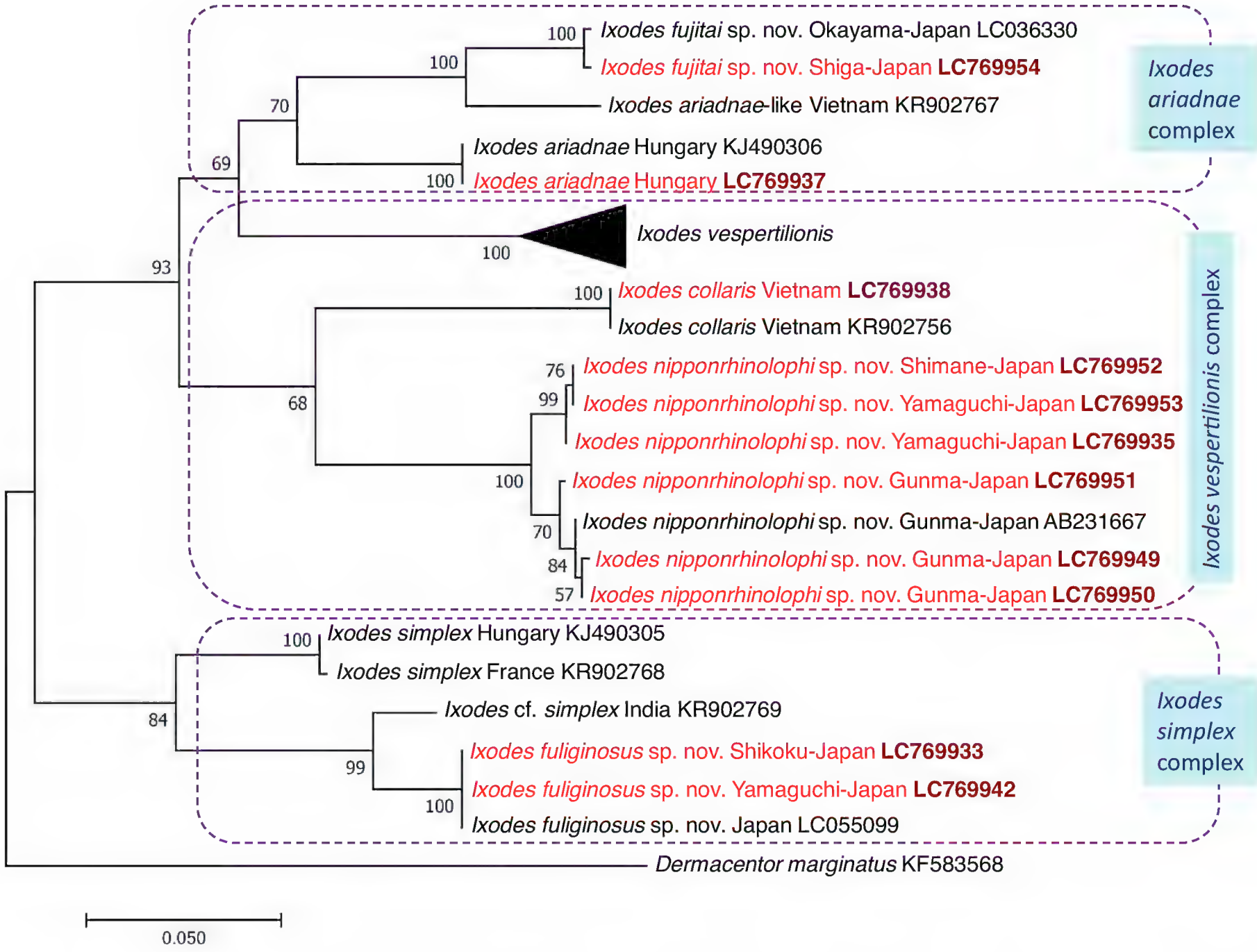
Ixodes fujitai sp. nov. Okayama-Japan LC036330
Ixodes fujitai sp. nov. Shiga-Japan LC769954
Ixodes ariadnae-like Vietnam KR902767
Ixodes ariadnae Hungary KJ490306
Ixodes ariadnae Hungary LC769937
Ixodes ariadnae complex
Ixodes vespertilionis
Ixodes collaris Vietnam LC769938
Ixodes collaris Vietnam KR902756
Ixodes nipponrhinolophi sp. nov. Shimane-Japan LC769952
Ixodes nipponrhinolophi sp. nov. Yamaguchi-Japan LC769953
Ixodes nipponrhinolophi sp. nov. Yamaguchi-Japan LC769935
Ixodes nipponrhinolophi sp. nov. Gunma-Japan LC769951
Ixodes nipponrhinolophi sp. nov. Gunma-Japan AB231667
Ixodes nipponrhinolophi sp. nov. Gunma-Japan LC769949
Ixodes nipponrhinolophi sp. nov. Gunma-Japan LC769950
Ixodes vespertilionis complex
Ixodes simplex Hungary KJ490305
Ixodes simplex France KR902768
Ixodes cf. simplex India KR902769
Ixodes fuliginosus sp. nov. Shikoku-Japan LC769933
Ixodes fuliginosus sp. nov. Yamaguchi-Japan LC769942
Ixodes fuliginosus sp. nov. Japan LC055099
Ixodes simplex complex
Dermacentor marginatus KF583568
0.050

Figure 8. Maximum Likelihood phylogenetic tree of bat-associated ticks based on the coxI gene. In each row of individual sequences, the region/country of origin and the GenBank accession number are shown after the species name. Rows of sequences from this study are indicated with red fonts and bold accession numbers. Nineteen sequences of *I. vespertilionis* were used (JX394205–JX394208; KJ490307–KJ490311; KR902757–KR902777) but their clade is shown collapsed (as a triangle).

Ixodes fuliginosus Hornok & Takano, sp. nov. was formerly considered *I. simplex*, with several similar and different characters noted above or shown between specimens from Europe and Eastern Asia: for instance, the anal groove was reported to be pointed in front of the anus in case of both *I. simplex* (Arthur 1956) and its variant in Japan (Yamaguti et al. 1971), so this is not part of their differential diagnosis. On the other hand, the front of basis capituli and areae porosae were reported to be important distinguishing characters between *Pholeoixodes* species (Hornok et al. 2017), which (according to the taxonomical requirement of monophyly) should include *Eschatocephalus*, thus *I. simplex* and *I. fuliginosus* Hornok & Takano, sp. nov. (Hornok et al. 2023). Relevant differences between *I. simplex* from Europe and Japan were already noted by Arthur (1956), i.e., focusing on the tectum, and the shapes of the basis capituli and areae porosae. Similarly, the number of posterior setae (hairs) is fewer, 8–12 on coxae IV in case of *I. simplex*, also shown or stated in previous descriptions and illustrations of this species (Babos and Janisch 1958; Hornok 2017), in contrast to the higher number (16–18) in *I. fuliginosus* Hornok & Takano, sp. nov.

Phylogenetically, the closest relative of *I. fuliginosus* sp. nov. is *I. simplex* (Fig. 8). Based on coxI sequences, the genetic divergence between these two species is 10–10.6% (60/601–66/623 bp) which exceeds (almost doubles) the average limit of interspecific boundary, 6.1% (Lv et al. 2014), thus confirming the taxonomical status of *I. fuliginosus* sp. nov. as a new species. The taxonomy of the preferred (specific) host of *I. simplex*, i.e., the Common Bent-wing bat, *Miniopterus schreibersii* (Kuhl, 1817) was revised, because previously it was thought to be a uniform species across Eurasia/Old World (Li et al. 2015). Although in this study *I. fuliginosus* Hornok & Takano, sp. nov. was more often collected from Big-footed myotis, *Myotis macrodactylus*, its main host is probably the Eastern Bent-wing bat, *M. fuliginosus* (as reported in Hornok et al. 2015b) which is distributed from the Caucasus to southern Asia including China, the Korean Peninsula, and Japan (Li et al. 2015). In line with this, the relatively low (~ 4%) coxI sequence difference of *I. fuliginosus* Hornok & Takano, sp. nov. from *I. simplex* reported from India (KR902774: Hornok et al. 2015b) suggests that *Ixodes fuliginosus* Hornok & Takano, sp. nov. has a broad geographical range in southern Asia.

Ixodes fujitai Hornok & Takano, sp. nov. is most similar morphologically to *I. ariadnae*, and phylogenetically belongs to a sister group of its specimens from Europe (Fig. 8). Based on coxI sequences, the genetic divergence between these two species is 10.3% (65/630 bp) which significantly exceeds the average limit of interspecific boundary, 6.1% (Lv et al. 2014), further supporting the taxonomical status of *Ixodes fujitai* Hornok & Takano, sp. nov. as a new species.

Importantly, Hilgendorf's Tube-nosed bat, *Murina hilgendorfi* (Murininae), the only hitherto known bat host of *I. fujitai* Hornok & Takano, sp. nov. (Hornok et al. 2015b; this study) is closely related to *Myotis* species (Ruedi et al. 2013), the preferred hosts of *I. ariadnae* (Hornok et al. 2014, 2016).

The results of phylogenetic analyses also justify that bat-associated ixodid tick species form complexes based on their general morphology, i.e., members of the *I. ariadnae* complex have short palps and long legs, those in the *I. vespertilionis* complex have long palps and long legs, whereas species of the *I. simplex* complex have short palps and short legs (Fig. 8). Based on the coxI gene, the *I. ariadnae* and the *I. simplex* complexes are monophyletic.

In conclusion, in the case of all three bat-associated tick species that are newly described in this study, the coxI sequence differences from formerly known members of their species complexes strongly support their status as separate species. In addition, previous data on the host preference of these groups were also confirmed, i.e., members of *I. ariadnae* complex were collected from vesper bats (Vespertilionidae), those in the *I. vespertilionis* complex mostly infest horseshoe bats (Rhinolophidae), and species of the *I. simplex* complex typically associate with bent-winged bats, Miniopteridae (Hornok et al. 2016). However, larger scale sampling will be necessary in the future to draw final conclusions in this respect.

As illustrated above, new ixodid tick species might exist and await discovery and description even in such exhaustively explored countries as Japan. Although ixodid bat ticks are relatively rare in the region of eastern Asia, further steps need to be taken to find and describe males and immature stages of these new species, as well as to ascertain their more complete geographical and host ranges.

Acknowledgements

The authors are grateful to K. Okumura, S. Ando (Shimane Nature Museum of Mt. Sanbe), F. Sato, K. Funakoshi, M. Yamada, and R. Kuwata (Okayama University of Science) for tick collection in Shiga, Shimane, Kanagawa, Kumamoto, Okayama, and Kochi, respectively. The authors also thank A. D. Sándor for providing an *I. simplex* female for digital photography, T. Murakami for cave guidance, and the late Dr H. Fujita for providing specimens of ticks. Last but not least, the authors highly appreciate that Ms. Ciara Reynolds (Virginia, USA) checked the text for English language.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This work was supported by AMED under grant number 22fk0108614h0602(AT), by the grant of the Research Center for Thermotolerant Microbial Resources, Yamaguchi University (AT), and by the Research Fellowship of JSPS KAKENHI under grant number JP21K05612(TY). SH received funding from Hungarian Research Network (HUN-REN), Hungary (Project No. 1500107).

Author contributions

Ai Takano: conceptualization, writing, data curation, methodology. Takeo Yamauchi: data curation, methodology. Mamoru Takahashi: data curation, methodology. Hiroshi Shimoda: data curation, methodology. Yasuhiro Gotoh: data curation, methodology. Junko Mizuno: data curation, methodology. Michio Natsume: data curation, methodology. Jenő Kontschán: data curation, methodology. Dávid Kováts: data curation, methodology. Vuong Tan Tu: data curation, methodology. Sándor Hornok: conceptualization, writing, data curation, methodology.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Collection data and accession numbers of specimens

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Link: https://doi.org/10.3897/zookeys.1180.108418.suppl1

Supplementary material 2

Additional morphological details of *Ixodes nipponrhinolophi* Hornok & Takano, sp. nov

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Data type: pdf

Explanation note: (**A**) dorsal habitus, (**B**) ventral habitus, (**C**) spiracular plate, (**D**) anal valves. Scale bars: 500 µm (**A, B**); 100 µm (**C, D**).

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Link: https://doi.org/10.3897/zookeys.1180.108418.suppl2

Supplementary material 3

Additional morphological details of *Ixodes collaris*

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Data type: pdf

Explanation note: (**A**) dorsal habitus, (**B**) ventral habitus, (**C**) spiracular plate, (**D**) anal valves. Scale bars: 1000 µm (**A, B**); 100 µm (**C, D**).

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Supplementary material 4

Additional morphological details of *Ixodes fuliginosus* Hornok & Takano, sp. nov.

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Data type: pdf

Explanation note: (**A**) dorsal habitus, (**B**) ventral habitus, (**C**) spiracular plate of holotype, (**D**) paratype. Numbers are referred to in the text where relevant structures are described. Scale bars: 100 μm.

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Supplementary material 5

Additional morphological details of *Ixodes simplex*

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Data type: pdf

Explanation note: (**A**) dorsal habitus, (**B**) ventral habitus, (**C**) spiracular plate, (**D**) anal valves, anal groove. Numbers are referred to in the text where relevant structures are described. Scale bars: 100 μm.

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